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Differential effects of hydrocortisone and TNF α on tight junction proteins in an in vitro model of the human blood-brain barrier

CAROLA FÖRSTER^{‡§}, MALGORZATA BUREK[‡], IGNACIO A. ROMERO[†],
BABETTE WEKSLER^{||}, PIERRE-OLIVIER COURAUD* AND DETLEV
DRENCKHAHN[‡]

[‡]University of Würzburg, Institute of Anatomy and Cell Biology, Koellikerstrasse 6, D-97070 Würzburg, Germany.

*Institut Cochin, Centre National de la Recherche Scientifique UMR 8104, Institut National de la Santé et de la Recherche Médicale (INSERM) U567, Université René Descartes, Paris, France

[†]Immunology and Cell Biology Group, Department of Biological Sciences, The Open University Walton Hall, Milton Keynes MK7 6AA, United Kingdom

^{||} Weill Medical College of Cornell University, New York, NY 10021

Running title:

Hydrocortisone induction of BBB properties in human adult brain endothelial cell line

§ address of correspondence:

PD Dr. Carola Förster

Institute of Anatomy and Cell Biology

University of Würzburg

Koellikerstrasse 6

D-97070 Würzburg

Tel. + 49-931-312706

Fax. + 49-931-312712

e-mail: carola.foerster@mail.uni-wuerzburg.de

Abstract

Homeostasis of the central nervous system (CNS) microenvironment is maintained by the blood-brain barrier (BBB) which regulates the transport of molecules from blood into brain and backwards. Many disorders change the functionality and integrity of the BBB. Glucocorticoids are being used successfully in the treatment of some disorders while their effects on others are questionable. In addition, conflicting results between clinical and experimental experience using animal models arose, so that the results of molecular studies in animal models need to be revisited in an appropriate in vitro model of the human BBB for more effective treatment strategies. Using the human brain microvascular endothelial cell line hCMEC/D3, the influence of glucocorticoids on the expression of barrier constituting adherens junction and tight junction transmembrane proteins (VE-cadherin, occludin, claudins) was investigated and compared to other established BBB models. In hCMEC/D3 cells the administration of glucocorticoids induced expression of the targets occludin 2.75 ± 0.04 -fold and claudin-5 up to 2.32 ± 0.11 -fold, which is likely to contribute to the more than threefold enhancement of transendothelial electrical resistance reflecting barrier tightness. Our analyses further provide direct evidence that the GC hydrocortisone prevents endothelial barrier breakdown in response to pro-inflammatory stimuli (TNF α administration), which could be demonstrated to be partly based on maintenance of occludin levels. Our studies strongly suggest stabilisation of BBB function as a mode of GC action on a molecular level in the human brain vasculature.

Introduction

Homeostasis of the central nervous system (CNS) microenvironment is essential for its normal function and is maintained by the blood-brain barrier (BBB) (Pardridge, 1988; Risau & Wolburg, 1990). The cell types composing the BBB are endothelial cells, pericytes and the end-feet of astrocytes. Among them, several recent studies have highlighted the importance of the brain endothelial cells to form the morphological correlative of the BBB in this modular organization: the permeability properties of the BBB reflect, to a major degree, the tightness of the intercellular junctions between brain microvascular endothelial cells (Rubin *et al.*, 1991). Tight junctions (TJ) seal the endothelial cell layer and are especially well developed in endothelia of the BBB, in contrast to blood vessels outside the CNS, the TJs of which are less elaborate and facilitate exchange of solutes and macromolecules and allow leukocyte trafficking (Simionescu & Simionescu, 1991). Two different classes of integral membrane proteins constitute the TJ strands, occludin and members of the claudin protein family (D'Atri & Citi, 2002). The claudins, which have been identified in brain microvascular endothelial cells include claudin-5, claudin-12 (Matter & Balda, 2003), claudin-1 (Liebner *et al.*, 2000) and claudin-3 (Wolburg *et al.*, 2003; Coisne *et al.*, 2005). Moreover, numerous studies have demonstrated conclusively that TJ formation depends very much on the VE-cadherin-based adherens junctions (Lampugnani *et al.*, 1995; Gumbiner, 1996).

Disruption of the blood-brain barrier (BBB) has been described as a crucial step of neuroinflammatory conditions including brain tumors, cerebral ischemia, meningitis, encephalitis, and multiple sclerosis (MS). Therapeutic strategies for several of these diseases include treatment with GC (Engelhardt, 2000; Qizilbash *et al.*, 2002) but a detailed understanding of their mechanism of action is still outstanding. GCs exert a

variety of beneficial effects under neuroinflammatory conditions by acting on immune cells, the microglia and the blood-brain barrier, but they do fail to improve cerebral edema following stroke or even show adverse effects like the induction of hypertension in chronic administration, so that there is a clear need to further elucidate their molecular mode of actions (Reichardt *et al.*, 2006). Effects of GCs like hydrocortisone (HC) are known to be mediated by the glucocorticoid receptor (GR) (Beato, 1989). GR can bind to specific DNA sequences (glucocorticoid-responsive element, GRE) in the 5'-flanking region of target genes and transactivate gene transcription (Beato, 1989). Despite great progress in the field, many questions concerning the mechanism of GCs remain unanswered, for example the contribution of genomic and non-genomic effects or the cell-type specificity of their action.

Barrier-tightening effects of GC treatment have been demonstrated for cerebral endothelial cells in vitro (Hoheisel *et al.*, 1998; Romero *et al.*, 2003; Förster *et al.*, 2005; Weksler *et al.*, 2005). Matching data supporting an important role for GC-mediated tightening of the BBB by junctional protein induction have been demonstrated in vivo in the mouse (Förster *et al.*, 2006). GCs have further been shown to effectively restore barrier in a rat model of MS (Paul & Bolton, 1995; Schmidt *et al.*, 2003). Moreover, using serial MRI recordings, a reduction in the number of enhancing lesions has been observed in patients suffering from optic neuritis and MS after high-dose GC treatment and in clinical studies (Grauer *et al.*, 2001).

Based on these effects, researchers have begun to use diverse GCs for the in vitro-differentiation of in vitro models of the BBB (Hoheisel *et al.*, 1998; Romero *et al.*,

2003; Förster *et al.*, 2005; Weksler *et al.*, 2005). In vitro BBB models are isolated endothelial cell culture systems from brain capillaries that allow for the study of BBB functionality, e.g. GC action, separated from feedback or indirect systems that operate in vivo. Several in vitro BBB systems have been developed and various procedures have been applied to isolate and culture brain microvascular endothelial cells. They differ with respect to e.g. isolation procedure, culture conditions, cell type and origin (tissue and species) and culture system. Such a diversity leads to in vitro BBB systems with different characteristics, which makes the comparison and transference of results between these systems problematic.

For the transference of results to therapy, it is a necessary step to characterize human brain endothelial cell features in response to GCs versus those from animal origin and to delineate the molecular targets of GC action also in the human system. There was no established immortalized human brain endothelial cell line so far that could be used as an adequate *in vitro*-model to investigate the effects of GCs on the differentiation and regulation of the blood brain barrier in humans. Due to this shortcoming, a transference of molecular mechanisms induced by GC administration in animal models to the human system has not yet been achieved. After establishing hCMEC/D3 as an immortalized validated human model of the blood brain barrier (Weksler *et al.*, 2005; Cucullo *et al.*, 2007) we can now focus onto analysing molecular mechanisms of GC action at the human blood brain barrier. In the present study, the in vitro expression of the GR and the cellular response to the pro-inflammatory cytokine TNF α and the GC HC was examined in hCMEC/D3 cells. Data are then compared to established GC-responsive brain endothelial cell lines from murine, rat or porcine origin characterised previously, and qualitative and quantitative differences are discussed. The future use of the cell culture model hCMEC/D3 might

help to understand and hopefully even treat neurological disease in a more effective way.

Methods

Chemicals. Hydrocortisone was purchased from Sigma, Taufkirchen, Germany. Collagen IV was purchased from Fluka, Taufkirchen, Germany.

Isolation and culture of cerebral endothelial cells. The immortalised human brain microvascular endothelial cell line hCMEC was generated as described (Weksler *et al.*, 2005). The human brain microvessel endothelial cell line hCMEC/D3 retains the morphological characteristics of primary brain endothelial cells and expresses specific brain endothelial markers and cell surface adhesion molecules (Weksler *et al.*, 2005). Moreover, it was recently demonstrated that, when cultured under flow conditions, these cells maintain in vitro the physiological permeability barrier properties of the BBB in situ even in the absence of abluminal astrocytes (Cucullo *et al.*, 2007): hCMEC/D3 cells do retain the expression of endothelial and BBB markers (Weksler *et al.*, 2005), hCMEC/D3 cells retain an aerobic metabolic pathway and exhibit an inflammatory response, i.e. barrier failure, extravasation of leukocytes of these cells (Cucullo *et al.*, 2007).

Cell Cultures. hCMEC/D3 were cultivated as described by Weksler *et al.* (Weksler *et al.*, 2005) with modifications: until confluence, cells were grown on collagen IV-coated flasks in EGM-2 medium from Clonetics (Cambrex BioScience, Workingham, UK) consisting of EBM-2 basal medium amended with 2.5 % FCS and the growth factors VEGF, IGF-1, EGF, basic FGF, heparin as well as ascorbate and gentamycin from the EGM-2 BulletKit according to the manufacturers recommendation as

previously described (Weksler *et al.*, 2005). Briefly, for EGM-2 preparation, 500 µl bFBE single aliquots and 125 µl of each, IGF1, ascorbic acid, VEGF, EGF, Heparin single aliquots were added to 500 ml EBM-2 basal medium as described in the manufacturers recommendation. As important modification to this protocol, no HC supplementation from the EGM-2 bullet kit was added to the growth medium. After reaching confluence, the medium was changed to a serum-reduced but hormone-supplemented cell differentiation medium: consisting of EBM-2 basal medium containing 0.25 % FCS and 100 nM HC, but without other added growth factors described for the growth medium.

All cultures were supplemented with 100 IU/ml penicillin and 100 mg/ml streptomycin (1% PEST). Cells were maintained in an atmosphere of 5.0% CO₂ / 95 % air and at 37 °C.

Electrophoresis and immunoblotting.

Cells were plated at a density of $2.5 \cdot 10^4$ cells/cm² in collagen IV-coated (Fluka, Taufkirchen, Germany) petri dishes and grown to confluence. At confluence, cells were maintained in FCS-reduced differentiation medium (see above) and treated with TNFα and HCs as indicated in the figure legends.

For western blot analyses, cells were then dissolved in Laemmli sample buffer (Laemmli, 1970) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 15 % gels).

Protein contents were quantified by protein estimation directly from SDS-PAGE loading buffer using 0.1 % (w/v) Amidoschwarz (AppliChem, Darmstadt, Germany) in 25 % (v/v) Methanol/ 5 % (v/v) acetic acid. For immunoblotting, proteins were transferred in Kyhse-Andersen transfer buffer (Kyhse-Andersen, 1984) to Hybond

nitrocellulose membranes (Amersham, Braunschweig, Germany) which were blocked with 10 % (w/v) low fat milk in phosphate buffered saline (PBS, pH 7.4) and incubated overnight at 4 °C with the respective primary antibody (in PBS plus 10 % low fat milk). The polyclonal rabbit antibodies against occludin, claudin-5 and claudin-1 were purchased from Zymed Laboratories, California, USA. The polyclonal rabbit antibody sc-8992 against hGR was purchased from Santa Cruz Biotechnologies, California, USA. The polyclonal rabbit antibodies against occludin, claudin-5, hGR and VE-cadherin were used at a dilution of 1:1000. As secondary antibody, horseradish peroxidase-labelled goat anti-rabbit IgG (Jackson Immuno Res. Lab., West Grove, PA, USA) was used diluted 1:3000 with PBS. The polyclonal goat antibody sc-6458 against VE-cadherin was purchased from Santa Cruz Biotechnologies, California, USA. As secondary antibody, horseradish peroxidase-labelled donkey anti-goat IgG (Jackson Immuno Res. Lab., West Grove, PA, USA) was used diluted 1:3000 with PBS. Bound immunoglobulins were visualised by the enhanced chemiluminescence technique (ECL, Amersham). Densitometric analysis using Scion Image Beta 4.02 (Scion Corp., MD, USA) was performed for quantitation.

Quantitative real-time RT-PCR

For real-time RT-PCR, cDNA was synthesized using iSCRIPT cDNA synthesis kit (BioRad) and 1 µg of RNA from hCMEC/D3 cells treated or untreated with TNF α and HC. Human occludin, claudin-1, claudin-3, claudin-5, VE-cadherin, and GAPDH primers were designed using the Primer Express Software (Applied Biosystems) and obtained from MWG Biotech. Real-time RT-PCR was performed using the SYBR[®]Green PCR Master Mix (Applied Biosystems). Primers occludin forward (5'-

TCC ATT GGC AAA GTG AAT GA-3'), occludin reverse (5' AGA GGT GCT CTT TTT GAA GG-3'), GAPDH forward (5'- GAG TCA ACG GAT TTG GTC GT-3'), GAPDH reverse (5'- GAT CTC GCT CCT GGA AGA TG-3'), claudin-1 forward (5'- CCG TTG GCA TGA AGT GTA TG-3'), claudin-1 reverse (5'- AAG GCA GAG AGA AGC AGC AG-3'), claudin-3 forward (5'- AAG GTG TAC GAC TCG CTG CT-3'), claudin-3 reverse (5'- AGT CCC GGA TAA TGG TGT TG-3'), claudin-5 forward (5'- GAG GCG TGC TCT ACC TGT TT-3'), claudin-5 reverse (5'- GCC AGG TAT GAG ATC GTG GT-3'), VE-cadherin forward (5'- GCC AGG TAT GAG ATC GTG GT-3'), VE-cadherin reverse (5'- GTG TCT TCA GGC ACG ACA AA-3'). The ABI PRISM 7300 SDS software (Relative quantification study) was used to determine the cycle threshold (Ct) for each reaction and gene expression was normalized to expression of the endogenous housekeeping gene, glyceraldehyde phosphate dehydrogenase (GAPDH) based on the the $2^{\Delta\Delta Ct}$ method (where Ct means threshold cycle).

Bioelectric and permeability assessments. Cells were plated on top of collagen IV-coated transwell chambers for 6-well plates (24 mm diameter, membrane material: polyethylene terephthalate (PET), 0.4 µm pores, pore density/cm² 1.6 x 10⁶) (Falcon, Heidelberg, Germany) at densities of 2.5 x 10⁴/ cm² cells per well. When they had reached confluence at day 5, the different experimental sets of cells were transferred to differentiation medium containing reduced amounts of FCS (see above) and treated with TNFα or HC as indicated.

Resistance measurement:

Transendothelial electrical resistance (TER) was measured using an assembly containing current-passing and voltage-measuring electrodes (volt ohm meter EVOM,

equipped with a STX2 "chopstick" electrode set, World-Precision Instruments Inc., New Haven, CT, USA). Resistances of blank filters were subtracted from those of filters with cells before final resistances (in $\Omega \cdot \text{cm}^2$) were calculated. All experiments were repeated at least 6 times.

FITC-dextran and fluorescein flux measurement: Confluent monolayers treated or untreated with HC (see figure legends) were washed in prewarmed HEPES buffer (10 mM HEPES, pH 7.2, 0,1 % BSA, 4.5 % glucose) and subsequently preincubated for 5 min in HEPES buffer at 37 °C. FITC-dextran (Sigma) were purified from unconjugated FITC by size exclusion chromatography (Biogel P2 Polyacryamide gel, BioRad, München, Germany). Paracellular flux measurement was started by adding to the upper chamber of the Transwell system 100 μl of 50 mg/ml of 4, 10, 70 or 150 kDa FITC-dextran or 100 μl of 5 mg/ml fluorescein, respectively, in HEPES buffer to a final concentration of 1 mg/ml FITC-dextran or 0.1 mg/ml fluorescein, to the upper chamber. Paracellular flux was assessed by taking 100 μl aliquots from the outer chamber every 15 min during the first 90 min of incubation. (FITC-dextran and fluorescein fluxes had initially been monitored over a duration of 360 min, however, at incubation times longer than 90 min, cytotoxic effects of the labeled compounds on hCMEC/D3 cells were occasionally observed by brightfield microscopy in preliminary experiments). Fluorescence was measured using a Wallac Victor2 fluorescence spectrophotometer (Perkin-Elmer, Überlingen, Germany) with excitation and emission at 485 nm and 535 nm, respectively. The fluorescein and FITC-dextran clearance through the monolayer was compared to control clearance of uncoated wells and the volume cleared was plotted against time and the slopes of the curves were fitted using linear least square regression to calculate the permeability coefficients (P_e) of the endothelial monolayer according to the method of van Bree (van Bree *et*

al., 1988) . Blank filters coated with collagen IV were shown to not significantly restrict the permeation of the larger dextrans.

Analysis and Statistics. Values for TER and gene expression were averaged to establish a single value for hCMEC/D3 cells under different experimental conditions. Throughout, averaged values were reported as means \pm standard error (SE). The indicated statistical test (Mann-Whitney U-test) was performed assuming significance for $P < 0.05$ (*), high statistical significance at $p < 0.001$ (**).

Results

HC responsiveness of hCMEC/D3 cells

In an effort to investigate HC-sensitivity of hCMEC/D3 cells we examined GC receptor (GR) expression in this cell line by RT-PCR analysis, Western blot and immunocytochemistry (Fig. 1). RT-PCR analysis showed a dose-dependent downregulation of GR transcript to 0.81 ± 0.06 -fold after 48 hours of treatment with 50 nM HC, and to 0.63 ± 0.1 -fold after 48 hours of treatment with 100 nM HC, respectively, in hCMEC/D3 cells (n=6) (Fig. 1A). In cell lysates from untreated hCMEC/D3 cells, there was a strong signal for GR protein detectable by Western blot analysis (Fig. 1B). HC supplementation for 48 hours at physiological concentration (100 nM) led to a ligand-dependent reduction in detectable GR protein to 83 ± 0.6 % of untreated cells as evaluated by densitometric analysis of Western blots (n = 3) (Fig. 1B). HC treatment further stimulated GR translocation from the cytosol to the nucleus as evaluated by immunocytochemistry of treated and untreated hCMEC/D3-cells (Fig. 1C): in untreated hCMEC/D3 cells, GR protein (green) was weakly detected as cytoplasmic staining (Fig 1C). Upon HC treatment, nuclear concentration of GR (green) was confirmed, visualised by propidium iodide nuclear counterstaining (red) (Fig. 1C): after HC treatment, fluorescence was concentrated in the nucleus in hCMEC/D3 cells as confirmed by the use of computer imaging software (Adobe Photoshop CS, Seattle, WA, USA) to merge the individual images for FITC-GR and propidium iodide counterstain to assess similarity of staining pattern.

Summarising protein expression data collected by western blot analysis and immunocytochemistry, we show that ligand-bound GR is translocated to the nucleus

in human BBB endothelial cells hCMEC/D3 as reported for classical GC-responsive cells (Beato & Klug, 2000).

Effects of HC on brain EC resistance and permeability

We were able to develop a serum-reduced but hormone-supplemented cell differentiation medium based on the media previously described for murine cEND brain microvascular endothelial cells (Förster *et al.*, 2005) and hCMEC/D3 cells (Weksler *et al.*, 2005): until confluence, cells were grown in EGM-2 medium from Clonetics (Cambrex BioScience, Workingham, UK) in the presence of 2.5 % FCS and the growth factors VEGF, IGF-1, EGF, basic FGF, heparin as well as ascorbate and gentamycin from the EGM-2 BulletKit as previously described (Weksler *et al.*, 2005) but without HC supplementation used in this reference. The amounts of growth factor single aliquots used for this study are detailed in the method section. After reaching confluency, the medium was changed to the following serum-reduced but hormone-supplemented cell differentiation medium: medium contained 0.25 % FCS and 100 nM HC, but was lacking other added growth factors.

HC supplementation of the serum-reduced cell differentiation medium led to a significant increase in TER across the hCMEC/D3 monolayer. TER in the absence of HC was $69 \pm 19.3 \Omega \times \text{cm}^2$ while HC administration caused a significant increase of TER to the range of $199 \pm 5 \Omega \times \text{cm}^2$ (Fig. 2A).

To further validate the hCMEC/D3 BBB model, the paracellular permeability of macromolecules like non-charged FITC-dextran of molecular masses 4 kDa, 10 kDa, 70 kDa, and 150 kDa and of fluorescein ($M_r = 300 \text{ Da}$) was tested by assessing

flux across the hCMEC/D3 monolayer after removal of unconjugated FITC from the uptake mix by size exclusion chromatography as previously described (Romero *et al.*, 2003; Förster *et al.*, 2005) (**Table 1**). For this, we determined the permeability coefficients using the slopes of the curves representing cleared volume versus time according to van Bree (van Bree *et al.*, 1988). The permeability of small macromolecules across the monolayer of hCMEC/D3 cells in differentiation medium (0.25 % FCS) supplemented with 100 nM HC was significantly decreased over 90 min for fluorescein (by 48 ± 6 %) and the for the smallest FITC-dextran (4-kDa) (by 37 ± 4 %) comparative to control cells maintained in HC-free medium containing 0.25 % FCS (n = 6-8 filters, 3 independent assays). No significant decrease versus untreated cells was observed for the FITC-dextran 10-kDa, 70-kDa and 150-kDa (Table 1). According to the classical pore theory (van Bree *et al.*, 1988), one can relate the molecular radius of the solutes assessed (R) with their free diffusion coefficients (D) and the permeability coefficient Pe (**Table 1**). The calculation of the apparent pore size of the paracellular pathway revealed the existence of cylindrical pores of two apparent sizes, a large pore population with a radius of 92 ± 9 Å and a small pore population with a radius of 12.47 ± 0.45 Å within the hCMEC/D3 monolayer. Hydrocortisone treatment reduced the apparent pore size of the small pores to a radius of 7.9 ± 0.3 Å consistent with the reduced permeability of the monolayers following HC treatment. The calculation of the fractional porous area/path length as through the cell monolayer, i.e. A_p/dx , was calculated as a measure of steric hindrance through the pores in the D3 monolayer (**Table 1**). Results show that steric hindrance appears to be significantly reduced by hydrocortisone treatment for all the molecules tested as compared to untreated cells.

The presented data underline that HC-induced enhanced resistance to electrical current appears to be mainly caused by closure of small-sized pores, responsible for the passage of fluorescein and the smallest of the dextrans tested (4 kDa), but that other effects independent of the tight junction molecules seem to be additionally induced by HC treatment. Taken together, bioelectric and paracellular flux measurements demonstrate that these cells are an appropriate HC-responsive model to test HC effects on the human BBB.

Junctional protein expression in response to HC treatment in hCMEC/D3 cells

TJ and adherens junction proteins are known to be key mediators of blood brain barrier sealing and maintenance. In order to identify potential mechanisms of HC preservation of the blood brain barrier we therefore assessed changes in junctional protein levels (Fig. 3) and gene expression (Tab. 1). A positive effect of HC on barrier molecule expression of the cells could be verified for occludin and claudin-5. Claudin-5 contents were increased by 62 ± 22 % as compared to the untreated control, as assessed by western blot analysis and densitometric quantitation. Occludin contents were increased by 16 ± 7 % as compared to the untreated control. No effects were detected in the case of claudin-1 and VE-cadherin (Fig. 3).

Also by real time-RT-PCR analysis we could show that treatment with HC influenced gene expression of junctional protein encoding genes differently in a concentration-dependent manner (**Table 2**). As described previously for murine brain microvascular endothelial cells (Förster *et al.*, 2005; Förster *et al.*, 2006), treatment with HC induced occludin gene expression: control and HC-treated endothelial cells studied expressed occludin, occludin levels were however significantly increased in response to HC

treatment, with the effect being most pronounced at concentrations between 50 – 100 nM HC.

The claudins -1, -3, and -5 showed different responses to the HC treatment regimens: while claudin-5 gene expression showed a strong induction in response to 100 and 500 nM HC treatment, HC treatment did not lead to an increase in claudin-1 or claudin-3 levels as compared to untreated cells. For VE-cadherin, an upregulation of gene expression in response to HC was equally not observed, while treatment with the highest concentration (1000 nM) of HC even reduced gene expression in the case of claudin-1, claudin-3, claudin-5 and VE-cadherin, presumably due to cytotoxic effects of this supraphysiological concentration. The influence of HC on hCMEC/D3 cells was observed from 0.5 till 24 hours after the beginning of the treatment. We received most pronounced differences in gene expression after a duration of eight hours, a longer treatment did not lead to any differences.

HC preservation of the endothelial barrier in response to TNF α treatment

MS is a very common chronic neurological disease which is characterized by the infiltration of the central nervous system by T-cells reactive against myelin across a compromised BBB (Schwartz & Kipnis, 2005). The inflammatory mediator TNF α seems to play a key role within the pathological processes of MS and experimental autoimmune encephalomyelitis (EAE) as an animal model of MS (Weber & Rieckmann, 1995; Korner & Sedgwick, 1996; Pan *et al.*, 1996). In order to identify functional changes in the brain endothelium, we assessed TER and barrier constituting junctional protein and mRNA levels in response to treatment with the pro-inflammatory cytokine TNF α and a combination of TNF α and HC treatment (Fig.

4, Table 3). In order to test whether HC treatment prevents a compromise of BBB function in response TNF α administration, we assessed TER of untreated monolayers kept in differentiation medium (0.25 % FCS) for 48 h, of monolayers treated for 48 h with HC in differentiation medium, and compared the values with monolayers kept in differentiation medium (0.25 % FCS) for 48 h and then additionally treated for 8h with TNF α or monolayers kept in HC-supplemented differentiation medium for 48 h and then additionally treated for 8h with TNF α (Fig. 4A). We were able to show that 8 hours TNF α treatment led to a reduction in TER from $74 \pm 12 \Omega \cdot \text{cm}^2$ in control cells, to $42 \pm 7 \Omega \cdot \text{cm}^2$ in TNF α -treated cells, while treatment with HC increased TER values to $324 \pm 33 \Omega \cdot \text{cm}^2$. Pretreatment with HC before TNF α administration effectively prevented barrier breakdown, TER values amounted to $157 \pm 27 \Omega \cdot \text{cm}^2$ (Fig. 4A).

In order to identify potential mechanisms of HC preservation of the blood brain barrier under pro-inflammatory conditions we assessed changes in junctional protein levels and gene expression (Fig. 4B, Table 3). A significant reduction in levels of the TJ proteins occludin and claudin-5 was observed by Western Blot and densitometric quantitation (Fig. 4B,C) which could contribute to the negative effect of TNF α on barrier properties. 8 h of TNF α -treatment caused a decrease in occludin protein to $75 \pm 1 \%$ of untreated cells. Pretreatment with HC before TNF α administration prevented occludin loss, we were able to detect $110 \pm 1.5 \%$ of occludin protein levels as compared to untreated cells. When treated with HC alone, occludin contents were significantly increased to $139 \pm 4 \%$ of control values. Comparably, 8 h of TNF α -treatment caused a decrease in claudin-5 protein to $57 \pm 1 \%$ as compared to the untreated control. Simultaneous administration of TNF α and HC did however not

prevent claudin-5 loss as effectively as in the case of occludin, we were still able to detect claudin-5 protein levels reduced to 79 ± 2 % of untreated cells. When treated with HC alone, claudin-5 contents changed significantly as compared to untreated cells (increased to 110 ± 1.5 % of control) (Fig. 4B). We also assessed whether the inflammatory cytokine or the HC would influence levels of the adherens junction protein VE-cadherin could however not detect significant changes under most treatments. Surprisingly, only HC treatment concomitant with TNF α -treatment led to an increase in detectable VE-cadherin protein (118 ± 5 % of control) 8 h after treatment (Fig. 4B,C).

Also by real time-RT-PCR analysis we could show that treatment with the inflammatory mediator TNF α alone or simultaneous treatment with HC influenced gene expression of junctional components differently (**Table 3**).

All the endothelial cells studied expressed occludin. Occludin mRNA levels were not reduced in response to 8 hours TNF α treatment. Co-treatment with TNF α / HC maintained occludin gene expression at the control level. As described previously (Förster *et al.*, 2005; Förster *et al.*, 2006), treatment with HC alone even stronger induced occludin gene expression. In contrast, the expression of claudin-5 and VE-cadherin was affected discordantly by 8 hours of TNF α treatment. While claudin-5 gene expression decreased in response to TNF α treatment, HC treatment led to a strong increase in claudin-5 levels as compared to untreated cells. Concomitant TNF α / HC treatment prevented a downregulation of gene expression and the amounts of claudin-5 transcript did significantly exceed the levels measured in control cells. The situation differed from observations in the case of VE-cadherin: a downregulation of gene expression in response to TNF α was equally observed and could be prevented

with simultaneous HC treatment while treatment with the GC HC alone did not significantly increase gene expression levels (Table 3).

The influence of $\text{TNF}\alpha$ on hCMEC/D3 cells was observed from 0.5 till 24 hours after the beginning of the treatment. We observed most pronounced differences in gene expression after a period of eight hours, a longer treatment did not lead to any differences.

Discussion:

Breakdown of the BBB is a key feature of neuroinflammatory conditions, such as MS, encephalitis, meningitis, brain tumors and cerebral ischemia (Hamann *et al.*, 1995; Rosenberg, 2002; Sellner & Leib, 2006). Therapeutic strategies for such diseases with impaired BBB function include treatment with GCs (Engelhardt, 2000) although the mechanism of GC action is still not precisely determined. Barrier tightening effects of GC treatment have so far been demonstrated in clinical studies (Grauer *et al.*, 2001), in vivo in animal models (Paul & Bolton, 1995; Schmidt *et al.*, 2003; Förster *et al.*, 2006), and in isolated cerebral endothelial cells from murine (Förster *et al.*, 2005) or rat (Romero *et al.*, 2003) origin identifying molecular targets for GC action at the cellular level. The verification of identified molecular GC targets in human cells has however not yet been achieved although necessary in order to apply results from diverse animal models to man and to further prospective therapeutic exploitation.

For the characterisation of hCMEC/D3 GC responsivity, we considered it appropriate to initially examine GR receptor status and barrier induction in this cell line. Our results indicated that human hCMEC/D3 cells express the GR. Our data further showed that GR message and protein in the presence of ligand is reduced in a concentration dependent manner. Remaining GR protein is translocated from the cytosol to the nucleus in hCMEC/D3 cells, as described for classical GC-responsive cells (Beato, 1989). Hormone-dependent down-regulation of steroid receptors, i.e. GR and the related progesterone receptor (PR), has been demonstrated in the past (Beato & Klug, 2000; Lange *et al.*, 2000) and might be necessary for the attenuation of transcriptional responses in tissues continually exposed to ligand (Shen *et al.*, 2001), a situation occurring precisely in the blood vessel-lining endothelial cells.

The GC HC was found to significantly induce barrier properties in the hCMEC/D3 BBB model. The maximal effect of HC was reached at a concentration of 100 nM or above, which is well in the physiological concentration range of HC in mammalian blood between 70 - 550 nM (Karlson *et al.*, 1994). GC treatment nearly tripled TER values across hCMEC/D3 monolayers up to $200 \Omega \cdot \text{cm}^2$. Treatment with HC further lowered the permeability of monolayers of hCMEC/D3 cells for fluorescein to 48 ± 6 % of untreated cells and for the smallest uncharged macromolecule (FITC-dextran 4 kDa) to 37 ± 4 % of untreated cells, while the permeability of higher molecular weight dextrans remained unaltered. The calculation of the apparent pore size of the paracellular pathway had revealed the existence of cylindrical pores of two apparent sizes, a large pore population with a radius of $92 \pm 9 \text{ \AA}$ and a small pore population with a radius of $12.47 \pm 0.45 \text{ \AA}$ within the hCMEC/D3 monolayer. Hydrocortisone treatment reduced the apparent pore size of the small pores to a radius of $7.9 \pm 0.3 \text{ \AA}$ consistent with the reduced permeability of the monolayers following HC treatment. Results further showed that steric hindrance as represented by the permeability to diffusion coefficient (P/D) appears to be significantly reduced by hydrocortisone treatment for all the molecules tested as compared to untreated cells. The values obtained for HC-treated cells hereby very closely match the data of van Bree who described the existence of longitudinal pores in brain endothelial cell monolayers from bovine origin (van Bree *et al.*, 1988). As an explanation, one might have to consider potential effects of HC on the endothelial cell glycocalyx in addition to the tight junction itself. Interestingly, Chappel *et al.* only recently reported such effects of hydrocortisone on the endothelial glycocalyx, the diminution of which increases capillary permeability, suggesting that the glycocalyx contributes to endothelial

permeability barrier formation and is yet another target for hydrocortisone action (Chappell *et al.*, 2007). Effects on the endothelial glycocalyx of D3 monolayers might thus have occurred independently of effects on tight junction molecules.

Future investigation will thus have to clarify the degree of pore size reduction and a possible reduction in pore number by HC treatment and have moreover to address so far unacknowledged effects on the endothelial glycocalyx.

Comparable effects on barrier tightness have been previously reported under HC treatment for the murine brain microvascular endothelial cell line cEND (Förster *et al.*, 2005; Förster *et al.*, 2006), primary porcine BCECs (Hoheisel *et al.*, 1998), rat brain microvascular endothelial cells (Romero *et al.*, 2003) and for cells of epithelial origin (Nguyen & Neville, 1998; Woo *et al.*, 2000). The data are further concordant with clinical reports describing the barrier closing effects of GCs on MRI gadolinium enhancement in acute demyelinating lesions (Burnham *et al.*, 1991) or in optical nerve neuritis (Grauer *et al.*, 2001).

In an attempt to elucidate the molecular targets of GC-induced tightening of the barrier, we were able to show that GC signals can directly act at the transcriptional level on human occludin and claudin-5 gene expression in a dose-dependent manner. This induction of gene expression was further reflected in increased occludin and claudin-5 protein synthesis, identifying them as molecular targets of GC action in human brain microvascular endothelial cells. Levels of another TJ protein, claudin-1, and the adherens junction protein, VE-cadherin remained unchanged by GC treatment.

These data are partly consistent and partly disparate from observations in animal models of the BBB, pointing to inter-species differences in GC responsivity: in the

mouse BBB model cEND, occludin could similarly be identified as a GR target (Förster *et al.*, 2005), while an effect on claudin-5 gene expression by GCs was not observed (Förster *et al.*, 2007). Induction of claudin-5 protein by GCs has however previously been described in other species than mouse: in rat brain microvessel endothelial cells (BMEC, GPNT), addition of HC to the cultures resulted in fewer frayed junctions and a more uniform distribution of the TJ protein claudin-5 at the cell borders as compared with cultures without GC treatment (Romero *et al.*, 2003; Calabria *et al.*, 2006). A recent screen of gene expression by human alveolar epithelial cells indicated that claudin-5 is one of the major genes upregulated during differentiation in culture (Gonzales *et al.*, 2002; Daugherty *et al.*, 2004). On the other hand, responsivity of the human VE-cadherin gene to GCs could not be observed in hCMEC/D3 cells, while it represents a GC target in the mouse (Blecharz *et al.*, 2007). Reports on GC effects on VE-cadherin in other species or tissues could not be found, while the epithelial adherens protein, E-cadherin was reported to be strongly induced by GC in human lung epithelial cell lines promoting the formation of an alveolo-capillary barrier in vitro (Hermanns *et al.*, 2004). In contrast to this, GCs did not modulate the production or location of the epithelial cell adhesion protein E-cadherin in mouse mammary epithelial cells in vitro (Zettl *et al.*, 1992). Whether this differential responsivity of the human, rat and mouse claudin-5 and VE-cadherin genes to GCs is based on different temporal gene expression patterns, the diverse GCs used or on different inter-species responsivity of the respective gene promoters to GCs remains to be investigated in the future. The discrepancy observed is however a strong indication to treat the transferability of results obtained in animal models to the human system with caution.

The contribution of individual TJ proteins on the development or progression of neurological conditions is varied. In some cases, TJ alterations and subsequent increased BBB permeability are an effect of the underlying pathology; alternatively, these changes can be causative and mediating event in disease development. For example, TJ disruptions and subsequent BBB perturbations are involved in the development of MS (Neuwelt, 2004; Brooks *et al.*, 2006), while ischemic stroke and traumatic brain injury lead to BBB perturbations (Ilzecka, 1996). There are many diseases, such as Alzheimer's disease, where the direct correlation is not yet known, but is currently being investigated (Wardlaw *et al.*, 2003). We thus attempted further to validate the hCMEC/D3 cell culture model suitable to study the barrier-preserving effects of HC under inflammatory conditions, and were able to demonstrate that TNF α treatment leads to divergent down-regulation of the different TJ proteins.

However, our study demonstrates that the simultaneous administration of HC with TNF α preserves the functional integrity of the TJs under inflammatory conditions (TNF α exposure). The selective down-regulation of the TJ proteins occludin and claudin-5 detected is congruent with other research and clinical reports concerning endothelial cells of the BBB (Brooks *et al.*, 2005; Förster *et al.*, 2005; Brooks *et al.*, 2006; Silwedel & Förster, 2006; Schreiber *et al.*, 2007) and non-neural endothelium (Zeissig *et al.*, 2007). Pre-treatment with HC maintained the TJ component occludin while it did not prevent the loss of claudin-5 and VE-cadherin as effectively. The observed effects suggest a vulnerability especially of claudin-5 protein to the pro-inflammatory cytokine. While the TJ transmembrane protein occludin seems to play a regulatory role in the control of vascular permeability, since tissue expression and content of occludin correlate well with barrier properties (Hirase *et al.*, 1997), the role of the different claudins appears to be strand and pore formation (Matter & Balda,

2003). Remarkably, differential effects of simultaneous HC/TNF α -administration on gene expression and protein levels of claudin-5 were observed: while no restoration of the claudin-5 protein levels could be observed, simultaneous HC/TNF α -treatment nevertheless led to increased claudin-5 gene expression. As an explanation, one might refer to previous observations, describing the upregulation of matrix metalloproteinases during inflammation in endothelial cells which demonstrably leads to the degradation of tight junction transmembrane proteins (MunBryce & Rosenberg, 1998) and could chiefly affect the claudin-5 based tight junction structural integrity at the protein level. In line with this hypothesis, Nitta et al. reported the identification of especially claudin-5 as a critical regulator of brain microvascular endothelial cell permeability (Nitta *et al.*, 2003). In retinal microvascular endothelial cells, Koto et al. could recently demonstrate that claudin-5 is a target molecule of hypoxia leading to the disruption of the barrier function (Koto *et al.*, 2007), while in a rat cortical cold injury model, Nag et al. were able to demonstrate decreased expression of both, occludin and claudin-5 at the site of injury (Nag *et al.*, 2007). TNF α and HC thus seem to act in a differential manner on the expression of different TJ components in microvascular cells of the BBB. Attention should however also be directed to the possibility that exposure to high levels of the proinflammatory cytokine TNF α can lead to increased amounts of locally produced VEGF from the endothelial cells, which will contribute to the downregulation of TJ proteins and increase leakiness of the barrier. HC effects could therefore also be indirect in nature by reducing VEGF secretion (Neuwelt, 2004). Future studies will thus have to address the nature of the opposed effects of HC and TNF α or other pro-inflammatory cytokines on TJ properties in brain microvascular endothelial cells, i.e. effects on TJ transcription, protein synthesis, intracellular localisation, phosphorylation status, on

the dynamics of junctional protein interactions, and on the secretion of signalling proteins and peptidases, in line with investigations in other cellular systems (Poritz *et al.*, 2004; Bruewer *et al.*, 2005; Prasad *et al.*, 2005; Abbott *et al.*, 2006; Förster *et al.*, 2007).

Taken together, our observations support the hypothesis that the secretion of pro-inflammatory cytokines during inflammation in endothelial cells leads to the degradation of chiefly claudin-5 and, to a minor extent, occludin, at the human BBB and could affect TJ structural integrity, while HC treatment is an effective way to prevent this. To the best of our knowledge, this is the first report describing a possible role of GCs like HC in direct regulation of the expression of TJ components in human brain microvascular endothelial cells. These observations might be of clinical and pharmacological significance, since it could open up new specific routes of treatment of CNS inflammatory diseases: at present, high dose GC therapy is used successfully in MS relapses. The prevailing opinion on the mode of GC action is that it induces the inhibition of cytokine-induced barrier reduction and expression of CAMs (VCAM-1, ICAM-1, E-selectin and PECAM-1) which mediate T cell/ BBB interaction and consequently chronic leukocyte recruitment across the BBB. In contrast, our observations open up a new lead for an understanding of the beneficial effects of GC action in a therapeutic regime: An additional mode of GC action on brain microvascular endothelium appears to consist of tightening of the barrier which is likely to diminish leukocyte recruitment across the BBB. After a future identification of the recruited transcriptional co-regulator proteins for GR (Beato & Klug, 2000), the design of cell- or tissue-specific steroidal drugs might be attempted to treat MS patients without the severe side effects of classical GC therapy.”

Figure legends.

Fig. 1.

(A) After reaching confluence, hCMEC/D3 cells were treated with 50 nM and 100 nM hydrocortisone (HC) for 48 hours and GR mRNA expression was assessed. Treatment was repeated every 24 h. A downregulation of GR transcript to 0.81 ± 0.06 -fold after 48 hours of treatment with 50 nM HC, and to 0.63 ± 0.1 -fold after 48 hours of treatment with 100 nM HC, respectively, was observed in hCMEC/D3 cells.

(B) Confluent monolayers of hCMEC/D3 cells were grown in collagen-IV coated cell culture flasks in the presence of 100 nM HC as indicated. Cell lysates were analysed by western blot for GR protein contents. After 48 h of HC treatment of hCMEC/D3, a downregulation of GR protein to an estimated protein content of 83 ± 0.6 % of that in untreated cells occurred, (n = 3).

(C) Immunocytochemistry visualising the cellular localisation of GR protein in hCMEC/D3 endothelial cells maintained in serum-reduced medium (0.25 % FCS) “control” as compared to cells maintained in differentiation medium (0.25 % FCS, 110 nM HC) “HC”. GR stain (FITC = green), propidium iodide nuclear counterstain (red), and merged images (GR/PI) of GR immunofluorescence (green) and nuclei counterstained by propidium iodide (red). After 48 h of HC treatment a nuclear concentration of GR (green) in hCMEC/D3 cells was observed, visualised by propidium iodide nuclear counterstaining (red). The nuclear concentration of GR could be confirmed for HC treated hCMEC/D3 cells by the use of computer imaging software to merge the individual images for FITC-GR and propidium iodide counterstain to assess similarity of staining pattern.

The slides were analysed using a Zeiss Axioscop2 microscope. All pictures within each experiment were captured and manipulated identically with SpotAdvanced software and Adobe Photoshop. Bar in the lower panels indicates 20 μm for all panels.

Fig. 2.

HC induces barrier properties in hCMEC/D3 cells.

Influence of the addition of HC on the electrical barrier properties (TER) of hCMEC/D3 monolayers. Growth medium (2.5 % FCS) was changed after 5 days in culture to differentiation medium (0.25 % FCS, \pm additions) and analysis of the TER was performed after additional 48 h *in vitro*, while treatment was repeated every 24 h. Incubation medium: (control) with 0.25 % (v/v) FCS, without hydrocortisone; (HC) with 0.25 % (v/v) FCS, 100 nM HC; Data are given as mean \pm sd (n = 6).

Fig. 3. HC effects on junctional protein levels.

The influence of HC treatment on the junctional proteins occludin, claudin-1, claudin-5 and VE-cadherin was assessed by Western Blot and densitometric analysis: for this, cells were seeded on plastic cell culture flasks coated with collagen IV, treated for 48 h as as described above and indicated and subjected to SDS-gel electrophoresis and Western blotting. An increase in protein levels could be verified for occludin and claudin-5. Claudin-5 contents were increased by 62 ± 22 % as compared to the untreated control, as assessed by western blot analysis and and densitometric quantitation. Occludin contents were increased by 16 ± 7 % as compared to the untreated control. No treatment-related changes were detected in the cases of claudin-1 and VE-cadherin (Fig. 3).

Fig. 4: HC preservation of hCMEC/D3 barrier function under pro-inflammatory conditions

(A) GC treatment prevents a compromise of BBB function in response to $\text{TNF}\alpha$ administration for 8 hours: TER drops from $74 \pm 12 \Omega \cdot \text{cm}^2$ in control cells to $42 \pm 7 \Omega \cdot \text{cm}^2$ in $\text{TNF}\alpha$ -treated cells, while treatment with HC increased TER values to $324 \pm 33 \Omega \cdot \text{cm}^2$. Simultaneous administration of $\text{TNF}\alpha$ with HC effectively prevented barrier breakdown, TER values amounted to $157 \pm 27 \Omega \cdot \text{cm}^2$.

(B) hCMEC/D3 cells were grown in collagen IV-coated cell culture flasks to confluence for 5 d and thereafter maintained in differentiation medium containing various additions for an additional 8 h: 0.25 % FCS; 0.25 % FCS + 100 nM HC; 0.25 % FCS + 10 nM $\text{TNF}\alpha$; 0.25 % FCS + 100 nM HC + 10 nM $\text{TNF}\alpha$. After 8 h, cell lysates were prepared. Cell lysates were analysed by western blot for occludin, claudin-5, and VE-cadherin. 8 h of $\text{TNF}\alpha$ -treatment decreased occludin protein to 75 ± 1 % of untreated cells. Pretreatment with HC before $\text{TNF}\alpha$ administration increased occludin to 110 ± 1.5 % of untreated cells and prevented occludin loss. When treated with HC alone, occludin was significantly increased to 139 ± 4 % of control values. In contrast, 8 h of $\text{TNF}\alpha$ -treatment decreased claudin-5 protein to 57 ± 1 % of untreated cells. Simultaneous administration of $\text{TNF}\alpha$ and HC yielded reduced levels of claudin-5 to 79 ± 2 % of untreated cells. When treated with HC alone, claudin-5 increased to 110 ± 1.5 % of control (Fig. 4B). Levels of the adherens junction protein VE-cadherin did not show significant changes under most treatment regimes. However, HC treatment concomitant with $\text{TNF}\alpha$ -treatment led to an increase in detectable VE-cadherin protein (118 ± 5 % of control) 8 h after treatment.

(C) Densitometric evaluation of (B)

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Abbreviations

GR (glucocorticoid receptor); HC (hydrocortisone); GC (glucocorticoid); cEND (cerebral microvascular endothelial cell line); BCECs (brain microvascular endothelial cells); CNS (central nervous system); BBB (blood-brain barrier); BSA (bovine serum albumine); TER (transendothelial resistance); GRE (glucocorticoid-responsive element), BMEC (brain microvascular endothelial cells)

References

Fig. 1

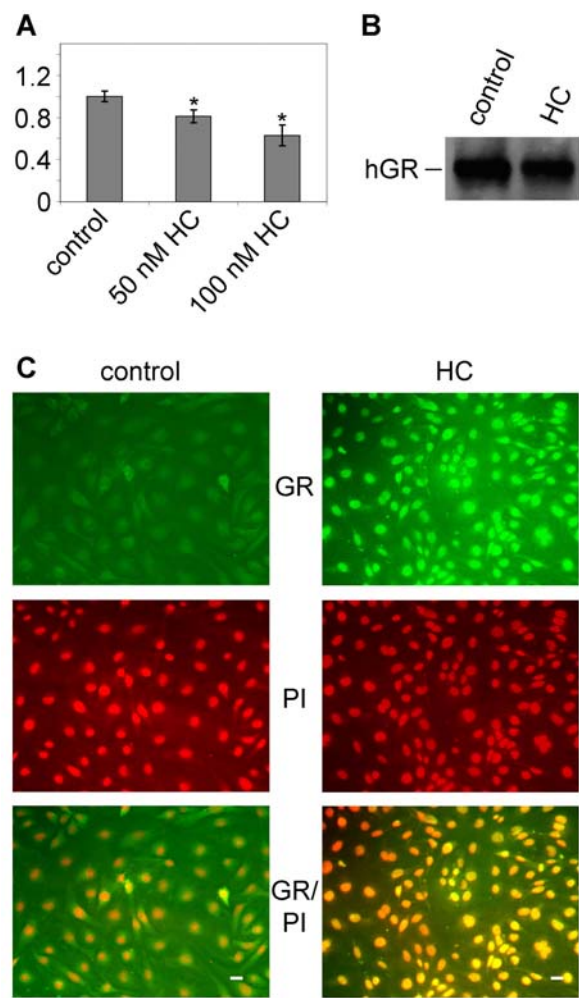


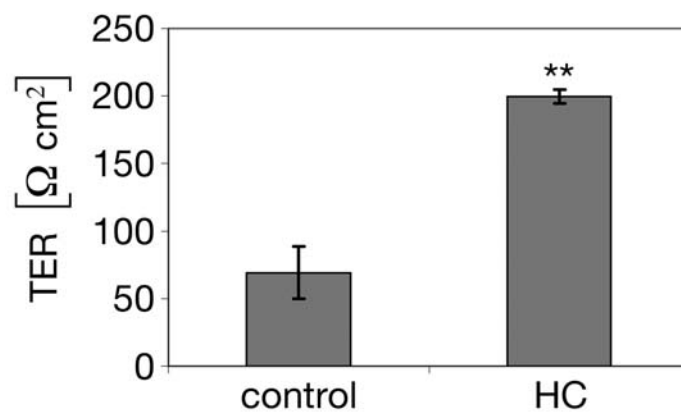
Fig. 2

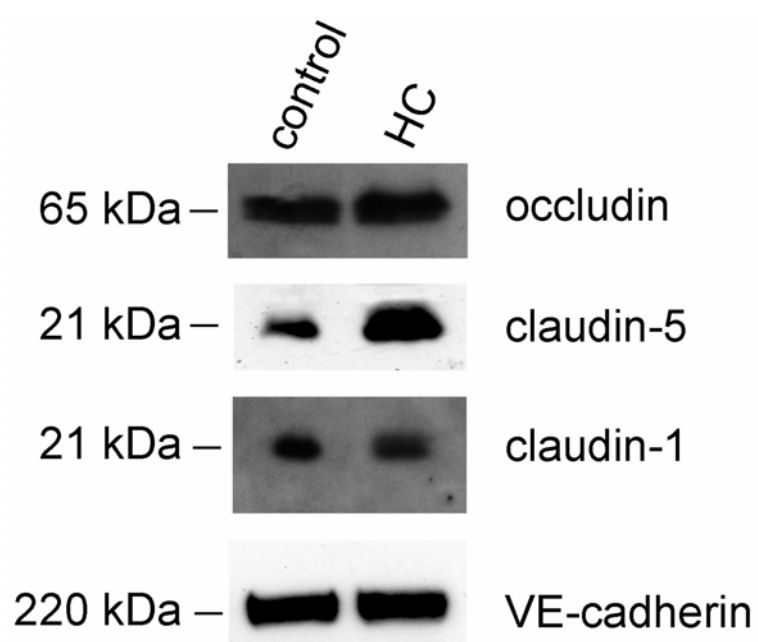
Fig. 3

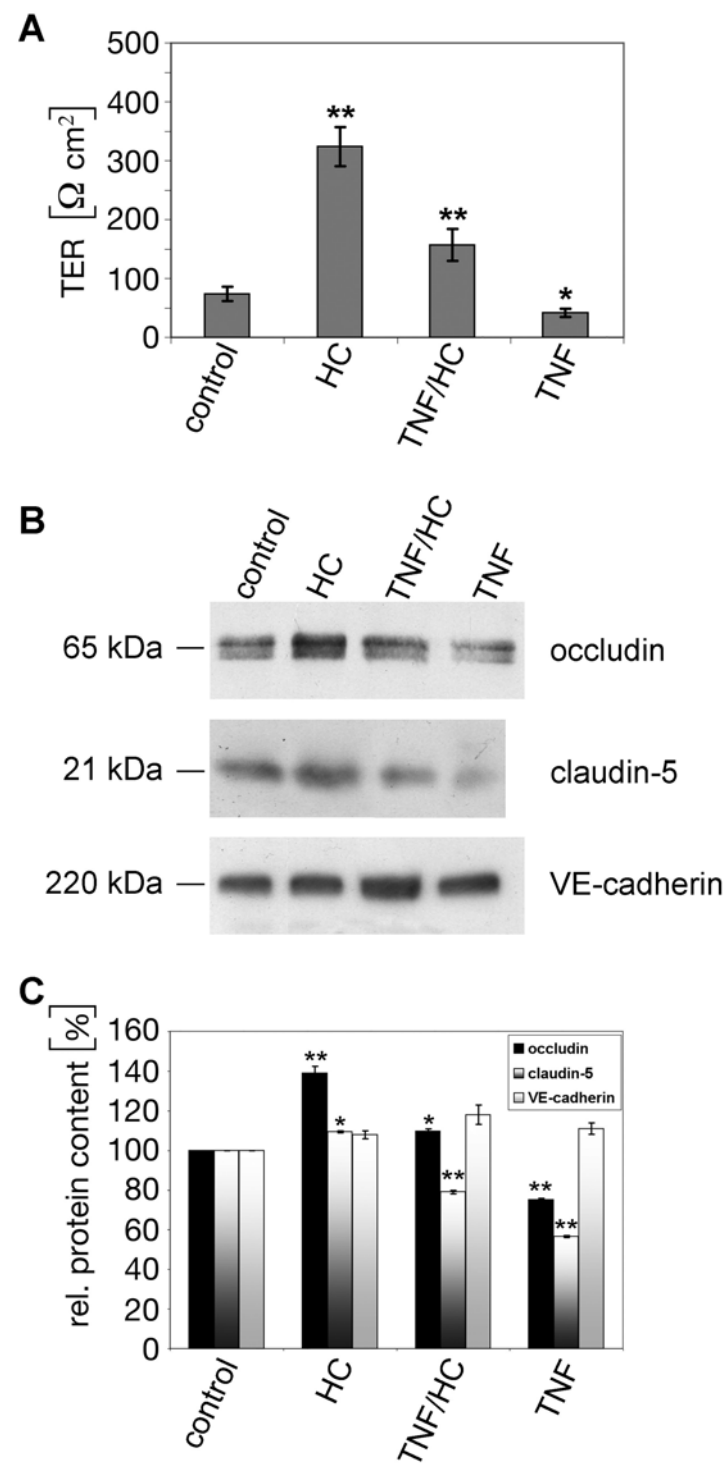
Fig. 4

Table 1

Permeability coefficients (Pe) for fluorescein and FITC-dextrans (4,10, 70 and 150) of control and HC-treated hCMEC/D3 cells (n = 6-8 filters, 3 independent assays).

Solute	MW	Pe (10 ⁻³ cm/min) control	Pe (10 ⁻³ cm/min) HC	Radius (Å)	D (10 ⁻⁵ cm ² /min)	Ap/dx (1/cm)	
						control	HC
Fluorescein	389	5.53 ± 0.07	3.43 ± 0.02	5.5	35.8	15.45	9.58
FD4	4000	0.83 ± 0.03	0.53 ± 0.02	14	14	5.93	3.79
FD10	10000	0.32 ± 0.02	0.28 ± 0.022	22	8.9	3.6	3.15
FD70	70000	0.017 ± 0.004	0.014 ± 0.004	60	3.3	0.52	0.42
FD150	150000	0.018 ± 0.006	0.014 ± 0.0035	80	2.5	0.72	0.56

FD, number = FITC dextran, molecular weight in kDa

D = Diffusion coefficient of the solute

A = Area

Pe = Permeability coefficient

Table 2

Modulation of TJ gene expression in hCMEC/D3 cells by the GC HC.

Gene/ statistical significance n = 5										
[nM HC]	occludin		claudin-5		claudin-1		claudin-3		VE-cadherin	
10	1.585±0.01	*	1.11±0.21	ns	0.89±0.14	ns	0.89±0.04	ns	0.87±0.03	ns
50	2.07±0.07	**	0.84±0.13	ns	0.81±0.04	ns	0.81±0.14	ns	0.98±0.14	ns
100	2.75±0.04	*	2.32±0.11	**	0.9±0.06	ns	0.9±0.27	ns	1.06±0.17	ns
500	1.79±0.54	**	1.88±0.06	*	0.77±0.01	*	0.77±0.31	ns	0.93±0.1	ns
1000	1.35±0.28	*	0.43±0.23	ns	0.64±0.07	*	0.635±0.2	*	0.75±0.2	ns

Fold expression versus untreated cells (means + s.d.), followed by statistical significance. *P<0.05, **P<0.001. ns, not significant. Values for untreated cells are set = 1. HC, hydrocortisone.

Table 3

Modulation of TJ gene expression in hCMEC/D3 cells by the inflammatory mediator TNF α and HC. Values for untreated cells are set = 1. n = 5.

-fold expression vs. untreated cells	occludin		VE-Cadherin		claudin-5	
TNF α	1.03 \pm 0.03		0.87 \pm 0.05	n.s.	0.71 \pm 0.09	*
HC	2.88 \pm 0.2	**	1.07 \pm 0.17	n.s.	2.84 \pm 0.14	**
TNF α /HC	1.2 \pm 0.14	*	1.4 \pm 0.36	n.s.	2.79 \pm 0.31	**.

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